

## Original Article

# MicroRNA-125-5p targeted CXCL13: a potential biomarker associated with immune thrombocytopenia

Jian-Qin Li<sup>1</sup>, Shao-Yan Hu<sup>1</sup>, Zhao-Yue Wang<sup>2</sup>, Jing Lin<sup>1</sup>, Su-Jian<sup>2</sup>, Yong-Chao Dong<sup>1</sup>, Xiao-Fang Wu<sup>1</sup>, Dai-Lan<sup>2</sup>, Li-Juan Cao<sup>2</sup>

<sup>1</sup>Department of Hematology, Soochow University Affiliated Children's Hospital, Suzhou 215003, Jiangsu, China;

<sup>2</sup>Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Suzhou 215016, Jiangsu, China

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**Abstract:** Background: Immune thrombocytopenia (ITP) is an acquired and autoimmune disease of adults and children characterized by decreased platelet production. CXC chemokine ligand-13 (CXCL13) participates in multiple immunological responses. However, it is still unknown the relationship between CXCL13 and ITP. Methods: Plasma CXCL13 was detected in ITP (n = 30) children. CD4+ T cells was isolated from peripheral blood mononuclear cells (PBMCs) from healthy volunteers. Treated CD4+ T cells with dexamethasone and/or miR-125-5p mimic/inhibitor, to observe the regulation of CXCL13. Results: Compared with controls, ITP children had elevated plasma CXCL13, the concentration of which was reduced after treatment. In vitro, dexamethasone decreased CXCL13 level in in dose-dependent and in time-dependent manner. MiR-125-5p mimic decreased CXCL13 level and miR-125-5p inhibitor increased CXCL13 level in CD4+ T cells. CXCL13 was implied to be target gene of miR-125-5p. MiR-125-5p inhibitor also canceled dexamethasone induced decrease of CXCL13. Conclusion: CXCL13 is the target gene of miR-125-5p, which is possibly involved in the pathological process of ITP.

**Keywords:** Immune thrombocytopenia, chemokine, lymphocytes, miR-125-5p

## Introduction

Immune thrombocytopenia (ITP) is an acquired and autoimmune disease of adults and children characterized by decreased platelet production. Childhood ITP is a large percentage of total ITP population [1]. Most ITP children recover within 6-12 months, and meta-analysis data shows that 20-25% children with peripheral blood platelet count  $< 100 \times 10^9/L$  lasting for more than 12 months will develop chronic disease [2]. Even so, digestive and urinary tracts bleeding, intracranial hemorrhage and subconjunctival hemorrhages are often accompanied with decreased platelet level and threaten the lives of patients [3, 4]. The pathogenesis of ITP remains not fully understood. However, most scholars consider humoral immune abnormalities leading to antiplatelet autoantibodies produced by B lymphocytes, which cause platelet damage, is the main reason for thrombocytopenia [5]. In addition, Tregs are decreased in num-

ber or exhibit defective suppressive functions in patients with ITP, which play critical roles in the maintenance of peripheral immune tolerance [6]. Early studies reported that platelet-reactive CD4+ T cells in ITP patients had an activity of promoting antiplatelet autoantibody response and were involved in the production of pathogenic antiplatelet autoantibodies in patients with ITP [7].

CXC chemokine ligand-13 (CXCL13) is a small cytokine belonging to the CXC chemokine family, mainly secreted by secondary lymphoid tissue, lymph gland and serum follicular dendritic cells [8]. The primary functions of CXC chemokine family are chemoattraction and activation of leukocytes in multiple immunological responses [9]. Chemokine receptor-ligand pair CXC chemokine receptor-5 (CXCR5) and CXCL13 is a key component in immune system [10, 11]. CXCL13 is required for B1 cell homing, natural antibody production and body cavity immunity

**Table 1.** Demographic, clinical, and laboratory data of children with ITP

Variables	No. Patients (%)
Age at the first presentation of ITP (y)	
< 1	6 (20.0)
1-3	7 (23.3)
3-7	8 (26.7)
> 7	9 (30.0)
Male	13 (43.3)
Hemorrhage severity	
None or mild	17 (56.7)
Moderate	8 (26.7)
Severe	5 (16.7)
Platelet count < 10 × 10 <sup>9</sup> /L at the time of hemorrhage	8 (26.7)
Hemoglobin concentration < 90 g/L at the time of hemorrhage	3 (10.0)
Type of ITP at last presentation of ITP	
Acute	20 (66.7)
Chronic	10 (33.3)

**Materials and methods**

*Collection of patient samples*

The human study was approved by Ethics Committee of Soochow University Affiliated Children's Hospital. We performed a retrospective review of children diagnosed with ITP in Soochow University Affiliated Children's Hospital (from January 2009 to December 2012). The medical records of all ITP children were collected: (1) general information: age and gender; (2) clinical data:

platelet count, hemoglobin concentration, severity of bleeding and type of ITP at last presentation of ITP. Severe hemorrhage was defined according to the diagnostic criteria of Bolton-Maggs and Moon's bleeding assessment tool [16]. IVIG, corticosteroids, splenectomy, immune therapy, and chemotherapy were included in the treatment of ITP.

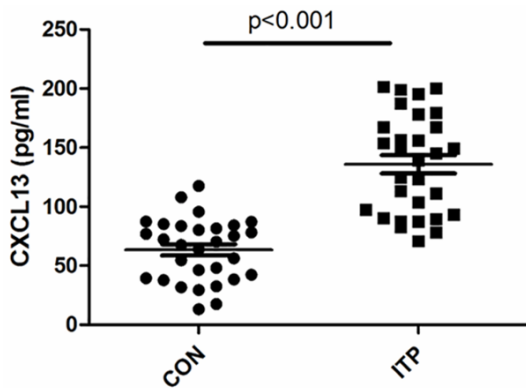
5 ml peripheral venous blood was collected by sterile syringes and then transferred to heparin sodium treated centrifugal tube from ITP children before and after treatment, and plasma was obtained by centrifuging at 3500 rpm for 10 min then stored at -80°C before biomarkers detection.

*Detection of protein CXCL13 by ELISA*

CXCL13 protein levels of plasma or cells were detected by ELISA kit (Shanghai Jining biological technology co., LTD, China) according to the manufacturer's instructions.

*Cell culture*

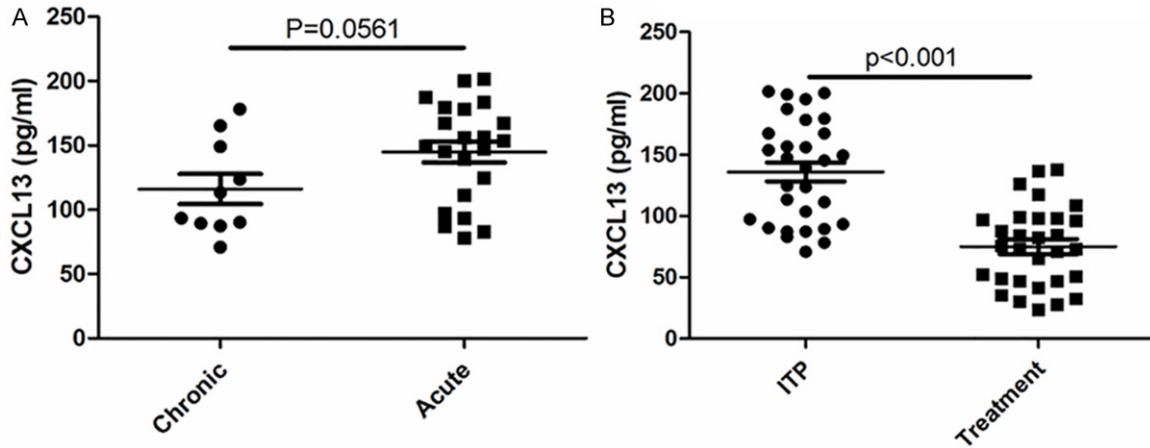
CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from healthy volunteers. As previously described [17], monocytes were depleted by adhesion for 30 min and CD4+ T cells were isolated by negative selection with magnetic beads using Automacs (Miltenyi Biotec, Germany) according to the



**Figure 1.** Plasma levels of CXCL13 in ITP and healthy children. Plasma CXCL13 was determined by ELISA kit. The concentration of CXCL13 was elevated in ITP children; n (CON) = 30; n (ITP) = 30.

[12]. In addition, it has been reported that CXCL13 plays a key role in recruitment of B cells and T-cell subsets in pathological conditions, and is considered to be a therapeutic target in various immune diseases [13, 14]. Research shows that CXCL13 level is elevated in ITP patients [15]. However, the relationship between CXCL13 and ITP is still unknown, and the regulation of CXCL13 in ITP remains to be further explored. In present study, plasma levels of CXCL13 in ITP children were determined and underlying mechanism was investigated in vitro.

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**Figure 2.** Plasma levels of CXCL13 in ITP children before and after treatment. Plasma CXCL13 was determined by ELISA kit; n (Chronic) = 10; n (Acute) = 20; n (ITP) = 30; n (Treatment) = 30.

**Table 2.** Univariate Correlations of plasma CXCL13 levels with disease activity parameters

	Before Treatment, rho (P)	After Treatment, rho (P)
Age	0.032 (0.871)	0.029 (0.916)
Gender	0.043 (0.932)	0.048 (0.908)
Hemorrhage severity	0.345 (0.021)	0.137 (0.318)
Platelet count	0.404 (0.002)	0.142 (0.412)
Hemoglobin concentration	0.298 (0.040)	0.083 (0.245)

manufacturer's instructions. CD4+ T Cells were incubated in complete RPMI 1640 medium containing 5% pooled human serum for 3 days. Inoculated cells to 24 well plates to culture. Treated cells with different concentrations of dexamethasone (Sigma, USA).

### Quantitative PCR

The total RNA of CD4+ T cells was isolated with TRIzol (Invitrogen, USA) and concentrated by isopropanol precipitation method. 2 $\mu$ g RNA per sample was reverse transcript to cDNA by M-MuLV Reverse Transcriptase (Promega, USA). The relative expression of CXCL13 mRNA and miR-125-5p was quantified by TaqMan Gene Expression Assays (Applied Biosystems, USA).  $\beta$ -actin and U6 were respectively served as internal control genes.

### Down-regulation and over-expression of miR-125-5p in cells

The expression of miR-125-5p in cells was regulated by transfected with mimic, inhibitor and

negative control of miR-125-5p (Ribobio Co., Ltd. China). The Lipofectamine 2000 reagent (Invitrogen, USA) was used to conduct cell transfection according to the manufacturer's instructions.

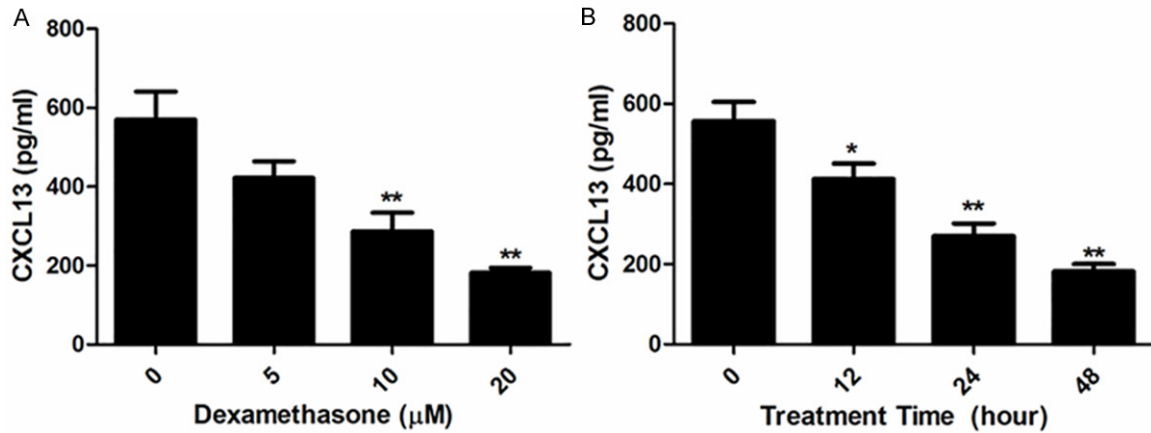
### 3'UTR luciferase assay

The 3'UTR luciferase assay was performed as previously described [18]. In brief, 3'UTR of CXCL13 containing puta-

tive miR-125a-5p binding sites were amplified by RT-PCR and then cloned into pMIR-REPORTTM vectors (Ribobio Co., Ltd. China). The constructs were co-transfected with pre-MIR-125a-5p or a negative control into 3T3 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Transfected cells for 48 h, the relative luciferase activity was determined using the Dual-Luciferase Reporter Assay System Kit (Promega, USA).

### Statistical analysis

Statistical analysis was conducted with SPSS18.0 software. Enumeration data was expressed as number (percentage). Measurement data was expressed as mean  $\pm$  SD. The univariate correlations of plasma CXCL13 levels with disease activity parameters were analyzed by Spearman method. The difference between two groups was determined by independent-samples T test. *P* value less than 0.05 was considered statistically significant.



**Figure 3.** CXCL13 decreased in CD4+ T cells treated with dexamethasone. CD4+ T cells was isolated from peripheral blood of healthy donors; different concentration of dexamethasone was treated cells for 24 h; 10 μM dexamethasone was used to treat the cells for different times; all experiments were repeated three times; \*p < 0.05 vs control; \*\*p < 0.01 vs control.

## Results

### *Demographic, clinical, and laboratory data of children*

There were 30 children diagnosed with ITP in this study (**Table 1**). The ratio of male to female was 1:1.3. The mean age of children diagnosed with ITP was  $5.4 \pm 3.7$  years. Of the 30 patients, 56.7% had none or mild hemorrhage, 26.7% ITP children had moderate hemorrhage and 16.7% suffered with severe hemorrhage. 8 (26.7) subjects had a platelet count  $< 10 \times 10^9/L$  and 3 (10.0) had a hemoglobin concentration  $< 90$  g/L at the time of hemorrhage. Of the 30 patients, 20 patients were diagnosed with acute ITP and 10 patients were chronic ITP.

### *Plasma levels of CXCL13 in ITP children*

CXCL13 concentration of peripheral blood was measured in ITP children and healthy children. As shown in **Figure 1**, ITP patients had a significant increase of plasma CXCL13 level compared with controls.

### *Plasma levels of CXCL13 in ITP children before and after treatment*

To compare the plasma CXCL13 level between chronic ITP and acute ITP, the difference was analyzed. As shown in **Figure 2A**, the CXCL concentration of acute ITP was higher than that of chronic ITP ( $P = 0.0561$ ). In addition, CXCL13 level was significantly decreased in ITP after

treatment (**Figure 2B**), which dropped to control level.

### *Univariate correlations of plasma CXCL13 levels with disease activity parameters*

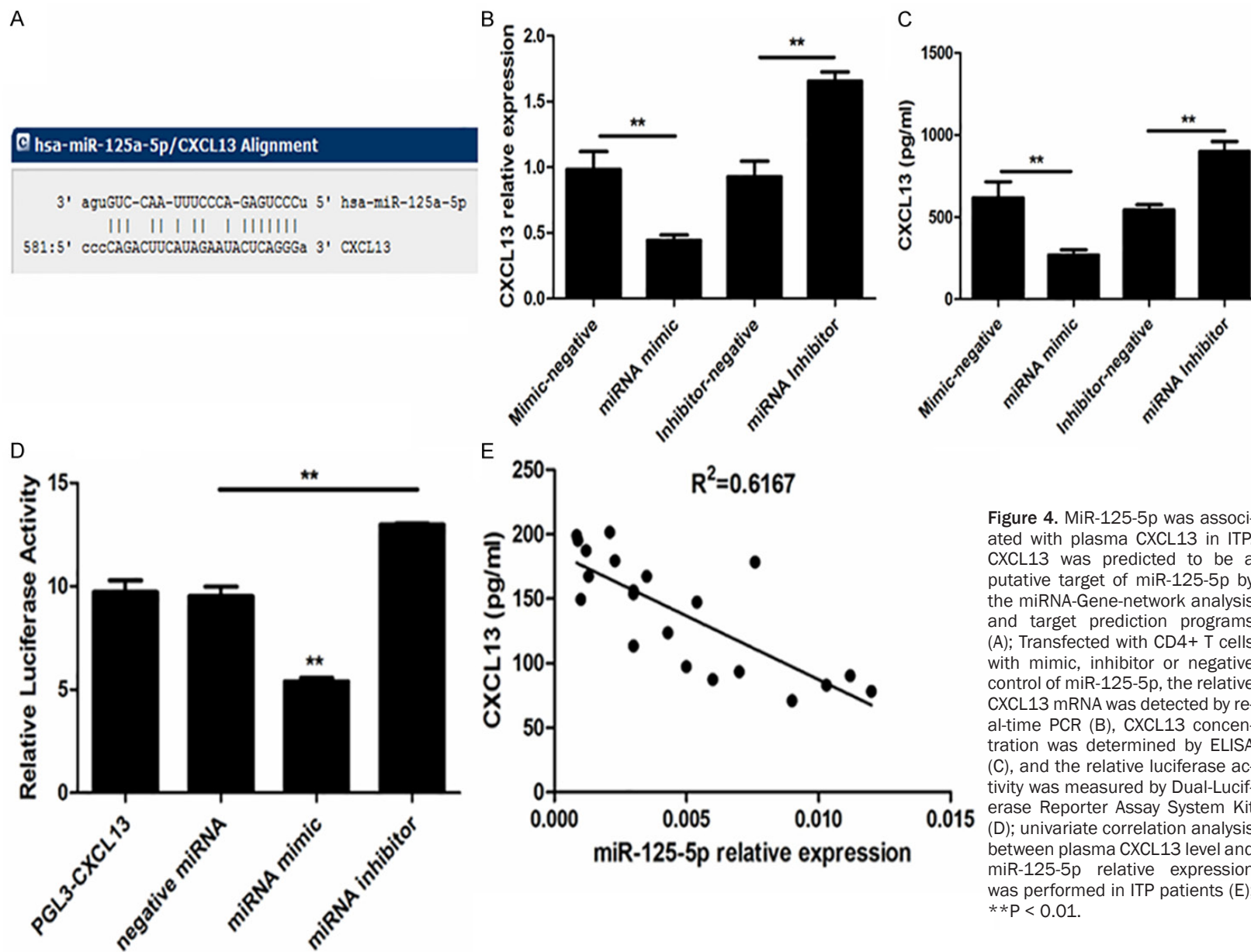
The correlation of plasma CXCL13 level and disease activity parameters were analyzed. As shown in **Table 2**, the CXCL13 level had no statistic correlation with age and gender in ITP children before or after treatment. The results showed that plasma CXCL13 level was positively correlated to hemorrhage severity, platelet count and hemoglobin concentration respectively in ITP patients before treatment. However, the correlations were absent after treatment.

### *CXCL13 decreased in CD4+ T cells treated with dexamethasone*

To further investigate the relationship between CXCL13 and ITP and its mechanism, *in vitro* experiments were performed. Dexamethasone was used to treat peripheral blood CD4+ T lymphocytes from healthy human in different concentrations and times of treatment. As presented, the CXCL level of CD4+ T lymphocytes was decreased in dose- dependent (**Figure 3A**) and time-dependent manner (**Figure 3B**).

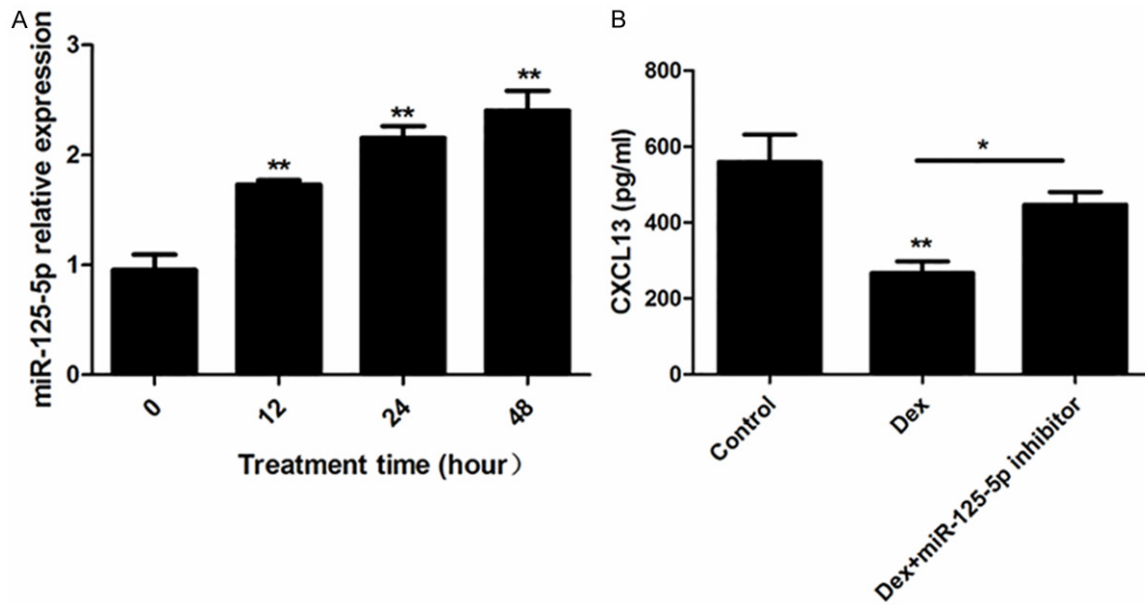
### *MiR-125-5p was associated with plasma CXCL13 in ITP*

CXCL13 was predicted to be a putative target of miR-125-5p by the miRNA-Gene-network analy-



**Figure 4.** MiR-125-5p was associated with plasma CXCL13 in ITP. CXCL13 was predicted to be a putative target of miR-125-5p by the miRNA-Gene-network analysis and target prediction programs (A); Transfected with CD4+ T cells with mimic, inhibitor or negative control of miR-125-5p, the relative CXCL13 mRNA was detected by real-time PCR (B), CXCL13 concentration was determined by ELISA (C), and the relative luciferase activity was measured by Dual-Luciferase Reporter Assay System Kit (D); univariate correlation analysis between plasma CXCL13 level and miR-125-5p relative expression was performed in ITP patients (E); \*\*P < 0.01.

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**Figure 5.** MiR-125-5p involved in the regulation of CXCL13 in CD4+ T cells. Treated CD4+MT cells with 10  $\mu$ M dexamethasone for different time, the miR-125-5p relative expression was detected by real-time PCR (A); CXCL13 concentration was measured by ELISA kit (B) after dexamethasone treatment with (or without) miR-125-5p inhibitor; \*P < 0.05; \*\*P < 0.01 vs control (or the group with 0 h treatment time).

sis and target prediction programs. The alignment was shown in **Figure 4A**. Transfected CD4+ T lymphocytes with mimic or inhibitor of miR-125-5p and incubated for 24h, the quantity of CXCL13 was detected by real-time PCR. The data showed that miR-125-5p mimic significantly down-regulated CXCL13 expression and miR-125-5p inhibitor markedly up-regulated CXCL13 level in CD4+ T lymphocytes (**Figure 4B**). Incubated cells for 48h after transfection, and the protein secretion of CXCL13 were quantified. Similar to the mRNA expression, CXCL13 protein secretion was decreased in miR-125-5p mimic and increased in miR-125-5p inhibitor (**Figure 4C**). Luciferase activity assay presented that miR-125-5p targeted to 3'UTR of CXCL13 (**Figure 4D**). The miR-125-5p expression of ITP patients was also quantified in peripheral blood mononuclear cells (PBMCs). As shown in **Figure 4E**, plasma CXCL13 was negatively correlated with PBMC miR-125-5p expression in ITP patients.

### *MiR-125-5p involved in the regulation of CXCL13 in T cell*

To further confirm whether the CXCL13 expression was regulated by miR-125-5p in treatment of dexamethasone, T lymphocytes were treated

with dexamethasone. The miR-125-5p level was increased in time-dependent manner in cells (**Figure 5A**). In addition, CXCL13 level was decreased by dexamethasone and miR-125-5p inhibitor reversed this effect.

### Discussion

Immune thrombocytopenia (ITP) is a common autoimmune disorder. Chemokine CXCL13 is key composition in immunity system. To investigate the relationship between CXCL13 and ITP, we determined the plasma CXCL13 in ITP *in vivo* first. The major findings showed that plasma CXCL13 was significantly elevated in ITP children. The subjects with acute ITP had higher CXCL13 level than that in chronic ITP. In addition, the patients after treatment had reduced plasma CXCL13 level compared with patients without treatment. Univariate correlation analysis result indicated that plasma CXCL13 level was positively correlated to hemorrhage severity, platelet count and hemoglobin concentration respectively in ITP patients before treatment. Our findings are in line with previous study which found CXCL13 is elevated in ITP patients [15]. All these data implied that CXCL13 was closely related to the development of ITP.

Chemokine CXCL13 is a key factor in establishing adaptive immune response. CXCR5 is the specific receptor for CXCL13. To mediate more effective response to secondary pathogen by memory T cells is a central process of adaptive immunity [19]. The pair of receptor-ligand (CXCL13/ CXCR5) greatly contributes to homing of B-helper cells and a subset of T-helper cells to the lymphoid follicle [20-22]. ITP is an immune-mediated acquired disease. Olsson B et al. revealed that activated T cells were apoptotic resistant in active ITP patients and lead to defective clearance of autoreactive T cells through apoptosis, which might cause a continued immune destruction [23]. CXCL13 may be involved in the pathophysiological procedure of ITP. However, few studies have been emerged to discuss the relationship between CXCL13 and ITP. As showed in our vivo experiments, plasma CXCL13 protein was elevated in ITP patients and positively related ( $r = 0.345$ ,  $p = 0.021$ ) to hemorrhage severity.

To investigate the mechanism of CXCL13 on ITP development, we performed experiments *in vitro*. Dexamethasone, a type of steroid medication that often used to treat ITP [24-26], was administrated into peripheral blood CD4+ T lymphocytes from healthy human, and results showed that CXCL13 in CD4+ T lymphocytes was decreased both in dose-dependent and in time-dependent manner. Therefore, *in vitro* experiments preliminary suggested that CXCL13 regulation participated in immune disorder.

MicroRNA is a small noncoding RNA molecule, taking part in various normal and pathological processes [27-29]. Jernas, M. et al identified 1915 regulated genes and 22 regulated microRNAs that differed between ITP patients and controls by genome-wide expression analyses in T cells. In addition, CXCL13 is demonstrated to be one target gene of miRNAs to regulate ITP [15]. CXCL13 was predicted to be a putative target of miR-125-5p by the miRNA-Gene-network analysis and target prediction programs. To better understand the mechanisms of CXCL13 involved in ITP, we over-expressed or down-regulated the miR-125-5p level in CD4+ T lymphocytes by mimic or inhibitor of miR-125-5p. As expected, over-expression of miR-125-5p decreased the mRNA and protein of CXCL13 while down-regulation of miR-125-5p increased the CXCL13 expression.

Luciferase activity assay proved miR-125-5p targeted to 3'UTR of CXCL13. All these results suggested that miR-125-5p played a key role in CXCL13 regulation. In another hand, miR-125-5p expression was time-dependently increased by dexamethasone treatment in CD4+ T lymphocytes. As described above, dexamethasone decreased CXCL13 protein level, and miR-125-5p inhibitor reversed this effect, which further confirmed that chemokine CXCL13 expression was regulated by miR-125-5p. There is evidence that miR-125a acts as an NF- $\kappa$ B inhibitor upon activation of TLR pathway and involves in inhibiting erythroid differentiation in leukemia [30]. Inoue, Y. found miR-125a was expressed in peripheral blood mononuclear cells (PBMCs) and the single nucleotide polymorphisms in precursor-miR-125a was associated with autoimmune thyroid diseases development and prognosis [31]. These previous studies imply that miR-125 is linked to immunological diseases. In present study, we also found miR-125-5p expression of PBMC was negatively correlated with plasma CXCL13 in ITP patients.

In conclusion, plasma CXCL13 level was significantly increased in ITP patients. *In vitro* experiments confirmed that CXCL13 is the target gene of miR-125-5p, the regulation of CXCL13 by miR-125-5p may be one of mechanisms in development of ITP. Further molecular mechanism of CXCL13 on ITP will be conducted. CXCL13 and miR-125-5p are potential therapeutic targets for ITP.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Jian-Qin Li, Department of Hematology, Soochow University Affiliated Children's Hospital, Suzhou 215003. No. 303 JingDe Road, Canglang District, Suzhou 215003, Jiangsu, China. Tel: +86 512 67787402. E-mail: JianQL023@163.com

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